Influence of hydraulic regimes and Cl_2/NH_3-N mass ratio on bacterial structure and composition in experimental flow cell chloraminated drinking water system

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Chloramine is the secondary disinfectant used within drinking water distribution systems (DWDS) for managing water quality. The growth of microorganisms is affected by operational conditions and the existance of these organisms in chloraminated systems can subsequently influence disinfection efficiency, particularly if nitrification process happens . In the current study, a next generation sequencing (NGS) analysis by Illumina MiSeq was applied to investigate the influence of different hydraulic conditions and Cl_2/NH_3-N mass ratios on bacterial structure and composition using an experimental temperature-controlled flow cell facility. The results obtained showed that the microbial community and structure was different between biofilm and water samples. *Actinobacteria* was the most dominant phyla within biofilm while *Alphaproteobacteria* was the most abundant in bulk water samples. There was no statistical difference in microbial community in biofilm identified between different hydraulic regimes, suggesting that biofilm is a stable matrix to outer environment. Results further showed that Cl_2/NH_3-N mass ratio had obvious effect on microbial structure in biofilm. This suggests that excessive ammonia is an influencing factor for microbial activity within biofilm. Within bulk water, species richness and diversity tended to be higher at lower hydraulic regimes. This confirms the influence of hydraulic condition on biofilm mechanical structure and further material mobilization to water. Opportunistic pathogens such as *Legionella* and *Mycobacterium* were detected in abundance in the experimental system. This confirms that nitrification can lead to decrease of water quality and microbial outbreaks. This research provides ecological information regarding hydraulic regimes and Cl_2/NH_3-N mass ratios influence on microbial structure and composition in experimental DWDSs experiencing nitrification process.

1. Introduction

Drinking water distribution system (DWDS) is the pipe system that transports finished water from treatment plant to the point of use. Before the water reaches customer taps, water quality deterioration can occur due to the influence of environmental conditions in the DWDS, and this is partly influenced by the system management[1,2] (i.e. hydraulic and disinfection) and water composition[3] (i.e. microorganisms and phisco-chemistry parameters). Of particular concern is the biofilm formation. Biofilm refers to a complex microbiological slime layer, composed of aggregated microbial cells and embedded within a self-produced matrix of extracellular polymeric substances (EPS) upon the pipe surface[4]. This matrix has stable structure and high resistance to external disturbance (i.e. detachment shear force and disinfection)[1,5]. Its characteristics, which includes density, structure and community, are affected by various abiotic and biotic properties, and subsequently impact on features of DWDS.

The presence of biofilm can lead to undesirable physical (pH, taste and odour, turbidity) and chemical (excessive ions, unexpected substance) changes in distribution system[6]. Moreover, since biofilm itself is a great shelter for potential pathogens, and coupled with the property to sorb water chemicals, growth of pipe scales and biofilm conglomerates is recognized as an underestimated source of contamination in DWDS[7,8]. In modem water treatment plants, chlorine and chloramine are the two main disinfectants used after the primary disinfection to maintain the water microbiologically safety by taking advantage of the disinfectant residual (free chlorine) they produce[9]. Compared with chlorine, chloramine has relatively low activity and produces less DBPs[10,11]. However, biofilm shows a resistance ability with chloramine and the concentration of disinfectant residual potentially decreased by nitrifiers[12,13]. In chloraminated DWDS, the free ammonia can provide substance for autotrophic ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), and ammonia can be converted to nitrite and then nitrate by these two kinds of nitrifiers respectively, referred to nitrification[14,15]. This reaction will not only reduce disinfectant efficacy but also bring about unintended impacts on pipe corrosion through inducing pH drop, and thereby increasing metal ion leaching[16]. Other impacts brought by nitrification can include increasing biofilm accumulation and escalating the possibility of regrowth events in distribution[17].

In order to provide advice about making efficient disinfection procedure in chloraminated DWDS, previous studies have focused on nitrifying bacteria properties and relevant influencing factors on nitrification[17-21]. Nitrifiers have low activity compared with heterotrophic bacteria and their growth relay on the available of inorganic substance (i.e. ammonia and nitrite)[22]. However, it is suggested that nitrifiers have preference to aggregate within biofilm[15,23] rather than live as free cells and hence their behaviour is not independent from the biofilm matrix. Without considering nitrification process and biofilm formation separately, the increasing

emphasis on water safety within DWDS with respect to disinfection efficiency of networks implies that it is important to understand and further control nitrification as well as biofilm together.

With the development of molecular technologies, researches have investigated the microbial community and structure within biofilm under various growth conditions[24,25]. The effects of different hydraulic regimes and disinfectants on biofilm formation, including the influence on microbial community have been investigated respectively[1,26]. However, for chloraminated DWDSs, especially those experiencing nitrification, no enough research has been done in terms of the relationship between microbial community and system operational conditions.

The overall aim of this paper is to investigate the impact of operational conditions, including hydraulic regimes and Cl_2/NH_3-N mass ratio on bacterial community composition and structure in biofilm and bulk water. This was achieved by applying Illumina MiSeq to analysis the microbial community within biofilm and bulk water samples collected from an experimental flow cell facility. In particular this study sought the understanding of the response of biofilm formed in chloraminated system experiencing nitrification to various hydraulic regimes and Cl_2/NH_3-N mass ratios. Such information is important to understand the role of biofilm and associated effect on nitrification process, so that to provide possible operational and management suggestions to the water utilities.

2. Materials and Methods

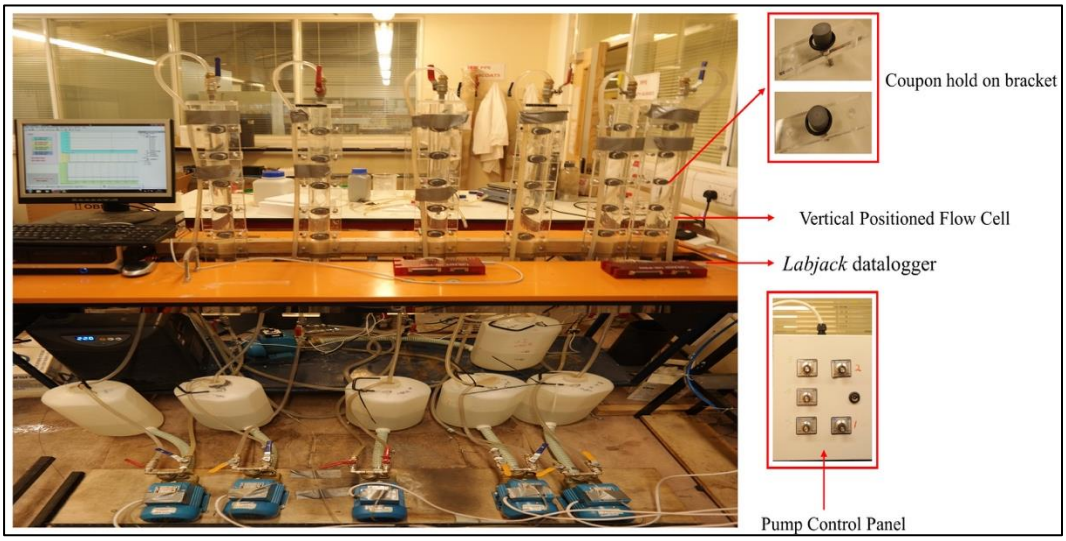

Figure 1 A series of flow cell systems in Characterisation Laboratories for Environmental Engineering Research laboratory at Cardiff University School of Engineering

2.1 Experimental facility and operating conditions

In the current study, a flow cell arrangement was applied to simulate the conditions of a pipeline within drinking water distribution systems (Fig.1). The characteristics of flow cell unit and design diagram were shown in Table S1 and Figure S1. The flow cell units were made of acrylic and had a length of 100cm. There are five equally spaced apertures along the planar surface of the flow cell, and these allow to fit 5 removable circular adhesion High-Density Polyethylene (HDPE) coupons, measuring 20mm in diameter. Due to the requirement for independent coupon position adjustment and convenient biofilm sampling, a separate holding bracket was used for holding the coupon (shown in Fig.1). The design allowed each coupon to be positioned perfectly with the internal surface of the flow cell, and the characterization of the composition of the in-situ biofilm assemblages quantitatively and qualitatively. During all experiment, the flow cells are positioned vertically to minimise trapped air within the system. A 0.33 kW centrifugal water pump (*Clarke* CEB 102) was used for water pumping and the flow rate in each cell was controlled independently by two ¼″ ball valves. The flow rate of each cell was monitored by an inline turbine flow meter (*RS* 511-4772). In order to avoid bias brought by temperature variation, the feed water was regulated by an external cooling unit (*D&D* DC-750) in order to maintain a constant temperature (16℃±1℃) within system. A *LabJack* multifunction 24-bit data logger (Model: U6-Pro) streamed all data recorded by a respective flowmeter to a desktop PC. DAQ factory (*AzeoTech*) data acquisition software was used to develop an interface to manage and export all measurement readings. The flow rate within each flow cell was monitored constantly and then maintained at a stable hydraulic condition.

In order to investigate how water quality changes under different operational conditions within chloraminated DWDSs experiencing nitrification, biofilm and nitrification process were developed before applying different operating conditions within the flow cell systems. In addition, the distribution and growth of biofilm was expected to be as even as possible, so as to minimize bias from biofilm distribution in later discussion. For this purpose, every discrete flow cell unit was connected in series and fed

with water from the same water tank. Fig.S2 shows the schematic diagram of the flow cells during this stage. At this stage, the system was fed with dechlorinated tap water containing high concentration of ammonia (50 mg/L NH_3-N) and adjusted to pH 8.0 with 5% (w/v) $NaHCO_3$. The pH and concentration of ammonia nitrogen, nitrite nitrogen and nitrate nitrogen were monitored all the time (shown in Fig.S4). Nitrification is considered to be established until a decrease of 50% of ammonia nitrogen within the feed water is observed. The nitrification establishment method outlined hereby was based on that described by Lee *et al.* (2011).

After a stable nitrification process was established and an even and well distributed biofilm was developed within all flow cells from the last stage, the facility was re-connected for the different experimental scenarios. The schematic diagram was shown on Fig.S3. For the experiments reported here two test phases were conducted. Total five different steady state hydraulic regimes were operated parallel in flow cells in both test phases, while two Cl_2/NH_3-N mass ratio (3:1 and 5:1) were applied respectively in these two phases.

Table 1 Experimental flow cell velocity, flow rate and determined boundary shear stress

Average flow rate Q (L/min)	*Average flow velocity U (m/s)*	*Reynolds number (Re)*	*Shear stress (N/m^2)*
2	0.05	1107	0.018
4	0.10	2214	0.036
6	0.15	3321	0.117
8	0.20	4428	0.194
10	0.25	5535	0.286

The water used in this experiment was collected from the tap located in CLEER lab (Cardiff University) and then stored in four 25 litre plastic water containers before been fed into the flow cell systems. Chlorine (from a stock solution of 500mg/L total chlorine) was then added into source water until a final concentration of approximately 1.0 mg Cl_2/L was achieved in all containers. After 24h, the chlorine in the containers was re-adjusted and ammonia (from pure ammonium chloride solids) was added (maintaining a total chlorine to total ammonia nitrogen ratio of 3:1 in test phase 1 or 5:1 in test phase 2) into the container until a chloramine residual (measured as total chlorine) of 1.0mg/L was achieved. Subsequently pH in the water was adjusted to 8.0 ± 0.1 after chloramination and from here on, this water will be referred to as the feed water. The five flow regimes were 2L/min, 4L/min, 6L/min, 8L/min and 10L/min, ranging from laminar to turbulent flow (1107<Re<5335). To ensure enough reaction time for presenting representative water quality change, the water age within the current study was maintained as three days. Table 1 shows the details of different hydraulic conditions in these two test phases. Each of the test phase was running for 33 days.

Before experiment commenced, the facility was disinfected with concentrated chlorine solution. The system was flushed for 48h at maximum flow rate (around 10L/min) and left to stand for another 24h after flushing. Fresh water was introduced to flush the system again at the maximum flow rate until the chlorine level became negligible. The insert coupons were sterilized with 80% ethanol solution for 24h and then left to dry in a clean fume cupboard for a further 24h. The above procedures were repeated before each test phase. The maintenance regime outlined hereby was based on that described by Douterelo *et al.* (2013) for a pilot DWDSs.

2.2 Sampling of biofilms and bulk water

To study the microbial community within the biofilm, coupons installed in every discrete flow cell (five for each) were collected after the two test phases. In order to remove the attached biofilm thoroughly, the coupon was immersed into 10ml sterilised phosphate-buffered saline (PBS) and then sonicated in an ultrasonic water bath (Kerry 2593) for 10 mins at approximately 50Hz. After all the five coupons have been washed, the suspension was centrifuged (*Eppendorf,* centrifuge 5424) at 14,000g for 2 mins to pellet the cells for DNA extraction and microbial analysis.

The water sample was collected from every discrete water tank after the tests. For every single flow cell unit, 1 litre of bulk water was taken directly from the tank and filtered through 0.22μm nitrocellulose membrane filters (Millipore, Corp). A total of 10 biofilm samples and 10 filters containing water samples were collected for subsequent DNA extraction and Miseq analysis.

2.3 Water Physico-chemistry

pH, DO and conductivity were constantly measured by using a benchtop meter (SevenExcellence S600) and probes. Several physico-chemistry parameters were monitored every three days during the tests. For each parameter, three subsamples were taken to increase the reliability and the average of the three replicates were calculated as the value used in this study. A HACH portable machine is used for turbidity analysis (HACH DR 900) based on standard method 2130 (APHA 1998). Total and free chlorine, nitrite nitrogen, nitrate nitrogen and ammonia nitrogen were measured using a Benchtop Spectrophotometer (DR3900, *Hach*-Lange) and relevant standard reagent assays (produced by *Hach* Lange). In particular, total and free chlorine concentrations were determined by N,N-diethyl-p-phenylenediamine (DPD) method (Method 8167 and 8021, HACH). Whereas, diazotization and cadmium reduction method kit were used to measure NO_2^- - N and NO_3^- - N respectively (Method 8507 and Method 8171, HACH). The ammonia nitrogen concentrations were measured using a Nessler reagent kit (Method 8038, HACH).

TOC was measured by a TOC analyser (TOC-V_{CPH} *Shimadzu*). The TOC concentration was estimated by determining the concentrations of total carbon (TC) and inorganic carbon (IC) (TOC = TC –IC). The TN was analysed by using the TOC analyser's TNM-1 accompanying unit.

2.4 DNA extraction and quantification

Based on the type of sample, the biofilm samples went through the extraction procedure immediately, whilst the filters with water samples required pre-treatment to remove the cells. In brief, the filter within 50ml centrifuge tube was washed by the filter wash buffer which was prepared by adding 6μl of Tween 20 to 3ml of PBS, and then mechanically shaken (*Lab Line,* Multi Wrist Shaker) for 10 mins. The cell suspension was transferred to a clean micro centrifuge tube and then the cells were pelleted by centrifuging the tube at 14,000g for 2 mins. The suspension was discarded.

For DNA extraction, the Metagenomic DNA Isolation Kit for water was used within the current study. In brief, the cell pellet was firstly re-suspended in 300μl TE buffer (10 mM Tris-HCl [pH 7.5], 1mM EDTA) and then the cell suspension had 2μl of Ready-Lyse Lysozyme solution and 1μl of RNase A added. After incubation at 37°C in water bath (*Julabo* TW12) for 30 mins, 300μl of Meta-Lysis Solution and 1μl of Proteinase K was added to the same tube and mixed by vortexing. 350μl of MPC Protein Precipitation Reagent was then added to the tube after cooling. After adding 570μl of isopropanol for precipitation, the DNA pellets was then washed by 500μl of 70% ethanol. Another centrifugation was followed by the washing step and the DNA pellet was dried and re-suspended in 50μl of TE buffer.

Qualification and quantification of extracted DNA were carried out, respectively on the TapeStation and Qubit. Agilent genomic DNA protocol was used for the Tapestation analysis, while the quantification process followed the Qubit assay. Thereafter, DNA in all samples was normalized to a final concentration of 15ng/μl and its quality was ~4.7.

2.5 16S rRNA sequencing with Illumina Miseq for characterising bacterial community

A dual-index sequencing strategy was performed on the Miseq Illumina sequencing platform for characterizing bacterial communities, and examining their relative abundance and diversity in water and biofilm samples. Extracted DNA was sent to the Heath Hospital, Cardiff University for bacterial 16S rRNA sequencing. One-step PCR amplification (30 cycles) was performed using the primers V4f and V4r[28] to construct 16S rRNA gene libraries. The sequencing procedure was described in detail by Kozich *et al.* (2013).

2.6 Sequence analysis

Within this study, a total of 63129~136456 valid 16S rRNA gene sequence were recovered from each biofilm and water sample through Illumina MiSeq sequencing analysis. With the obtained sequences, two independent analyses were undertaken by the Bioscience Department, Cardiff University. One of the analyses was aimed at obtaining taxonomical assignments from sequences and the other one was carried out to estimate alpha- and beta- diversity, which are two different terms to measure diversity in an ecosystem[29,30]. Alpha- diversity is about the diversity of a specific sample (i.e. how many different bacteria are in a sample), while beta- diversity refers to the difference between samples.

2.6.1 Mothur taxonomic analysis

Within the current study, Mothur (Version1.38.1) which is a custom Perl and C++ software, was used to take paired-end Illumina sequence reads, discover associated taxonomy and create a matrix of the count of each sequence in each sample. The pipeline required to implement the analysis are within the mother software package and specified on the mother website (http://www.mothur.org/wiki/Miseq_SOP) [31]. Following the method presented by Kozich *et al.* (2013), contigs that have any ambiguous bases (i.e., N), a homo-polymer run of more than 7 of the same base, or was shorter than 245 or longer than 275bp were removed. Subsequently, the sequence size was reduced to 2,334,474 by looking for contigs with identical sequence. The sequences were further aligned to reference alignment (Silva.Bacterial.fasta). Poorly aligned sequences were then removed and alignment was trimmed to remove positions that are not informative[32-34]. The sequences were trimmed to the ends to have them all start and end at the same alignment coordinates[34]. In order to further remove duplicate sequences within each sample, a pre-clustering algorithm was applied after identifying the unique sequences and their frequency[35]. UCHIME[36] was utilized for screening PCR chimeras within resulting sequences. The sequence was then classified by the Bayesian classifier against the Ribosomal Database Project (RDP) 16S rRNA gene training set (version 9). Sequences were removed if they did not classify to the level of kingdom or were classified as *Archaea, Eukaryota,* chloroplasts, or mitochondria. Finally, sequences were clustered into operational taxonomic units (OTU) at a 0.03 dissimilarity level and then a data matrix of each OTU in each sample as well as its abundance was made. The chimeras were identified based on mock community data and the sequencing error rates were calculated based on the method described by Schloss *et al.* (2011).

2.6.2 Alpha- and Beta- diversity analysis with *R*

Before the estimation of alpha- and beta diversity, each OTU was classified to get consensus taxonomy and the distance between sequences. A *biom* formatted file was then made for import into R software (Version 3.3.2). The sequences were representatives for each OTU subsequently. A phylogenetic tree was built using the FastTree algorithm[37] for UniFrac distance matrix construction.

With the R software, a phyloseq package was introduced for diversity analysis within the current study. To study the alpha-diversity (diversity within samples), a rarefaction analysis was performed for each sample based on hydraulic regimes, habitat type and test phases. Two different alpha-diversity metrics were included, which are Chao1 richness estimator[38] and Shannon diversity index[39]. To compare bacterial diversity between samples (beta-diversity), Unifrac distance metric was applied[40] to calculate

pairwise distance between communities in terms of their evolutionary history. Both un-weighted (presence/absence information) and weighted (considering relative abundance of each OTU) UniFrac analysis were undertaken.

2.7 Statistical analysis

To assess the similarity of community within different samples, the Bray-Curtis similarity matrixes were introduced using the R software (Version 3.3.2). The multiple-dimensional scaling (MDS) diagrams was used to have the matrixes visualised. A DEseq2 package (Version 1.16.1)[41], which use the negative binominal generalized linear model, was applied for determining the significant difference between biofilm and bulk water community.

In order to investigate the relationships between the water physico-chemistry variables and relative sequence abundance at class level within the biofilm samples, non-parametric Spearman's rank correlation coefficients were calculated using PASW Statistics 18.SPSS.

3. Results

3.1 Water physico-chemistry

As shown in Table 2, pH value was maintained at weakly alkaline conditions (7.54~8.28) for all the flow cell units within the two test phases. Free chlorine level dropped significantly due to the disinfectant decay. The concentration of nitrite nitrogen, TOC and turbidity all increased after the two tests for each hydraulic regime. Due to on-going nitrification in the simulated experimental facility, the level of ammonia nitrogen and TN declined in most of the cases. Turbidity was found to be higher for flow cells running with higher flow rates.

3.2 Correlation between physico-chemistry parameters and relative sequence abundance within biofilm

As shown in Table 3, there was no significant correlation between most of the bulk water quality parameters and the relative sequence abundance (RSA)[1] within biofilm, and only a positive correlation identified with the concentration of ammonia nitrogen ($p < 0.05$). However, significant correlations were observed between several water quality parameters. pH, nitrate-N and total nitrogen (TN) were strongly positively correlated with each other ($p < 0.01$). Ammonia-N was also significantly positively correlated with turbidity and total organic carbon (TOC) ($p < 0.01$).

3.3 Biofilm and bulk water bacterial diversity

As can be seen from Fig.2, the dominant bacterial phyla within the biofilms, was *Actinobacteria* followed by *Alphaproteobacteria, Betaproteobacteria, Planctomycetia, Gammaproteobacteria* and *Cytophagia*. The percentage of each of these bacterial groups varied depending on the particular hydraulic regime and disinfection strategies. These bacteria were also found in the bulk water samples, though with different abundance. Within bulk water, *Alphaproteobacteria* clearly dominated the bacterial community composition (average of total number of samples up to 46%) and to a lesser extent *Betaproteobacteria*, *Actinobacteria* and *Sphingobacteriia* were also abundant (Fig.2). At genus level (Fig.3), *Mycobacterium*, *Gemmata, Legionella* and *Azospira* were predominant within biofilms and *Mycobacterium, Sphingomonas, Sphingobium, Legionella, Flavisolibacter* and *Porphyrobacter* within bulk water samples (Fig.3). It should be noted that within the genus detected in the current study, *Mycobacterium, Legionella* and *Sphingomonas* were all considered as opportunistic pathogens.

Table 2 Physico-chemical properties of bulk water from the flow cell facility before and after tests

	Flow rate (L/min)	pH	Shear (N/m^2)	Turbidity (NTU)	Free Cl_2 (mg/L)	NO_2^--N (mg/L)	NH_3-N (mg/L)	NO_3^--N (mg/L)	TOC (mg/L)	TN (mg/L)
Test phase 1 (Cl_2/NH_3-N=3:1)	2	8.14	0.05	0	0.72	0.006	0.31	1.2	2.38	2.119
		8.00		4	0.08	0.021	0.09	0.7	2.98	1.208
	4	8.16	0.10	0	0.81	0.006	0.31	1.3	2.18	2.119
		7.79		7	0.10	0.015	0.20	0.4	3.20	0.5447
	6	8.23	0.15	0	0.90	0.009	0.30	1.5	2.85	2.214
		8.11		17	0.13	0.042	0.27	0.7	3.97	1.097
	8	8.27	0.20	0	1.08	0.009	0.30	1.4	1.19	2.111
		8.22		6	0.05	0.018	0.05	0.8	2.45	1.636
	10	8.28	0.25	0	0.98	0.008	0.30	1.5	2.72	2.164
		7.96		28	0.16	0.038	0.32	0.7	3.70	0.8738
Test phase 2 (Cl_2/NH_3-N=5:1)	2	7.65	0.05	0	0.49	0.005	0.20	1.0	2.22	1.638
		7.89		1	0.02	0.010	0.04	0.3	4.09	0.248
	4	7.72	0.10	0	0.50	0.004	0.24	1.0	2.40	1.743
		7.83		4	0.03	0.012	0.05	0.4	3.90	0.2645
	6	7.65	0.15	0	0.50	0.005	0.21	0.9	2.35	1.777
		7.88		19	0.11	0.013	0.32	0.5	6.93	0.9285
	8	7.54	0.20	0	0.64	0.003	0.20	1.1	2.16	1.639
		7.77		5	0.03	0.004	0.06	0.3	3.90	0.2789
	10	7.80	0.25	0	0.75	0.004	0.23	1.2	2.23	1.852
		7.93		11	0.09	0.021	0.11	0.8	6.11	0.8888

Corresponding to each flow rate, the above line was data collected before test and below one was measured after test.

Table 3 Spearman's correlation coefficients for water physico-chemistry factors and the percentage of relative sequence abundance at class level within biofilms

	Biofilm						
	RSA	**pH**	**Shear**	**Turbidity**	**Nitrite-N**	**Ammonia-N**	**Nitrate-N**

pH	NS						
Shear	NS	NS					
Turbidity	NS	NS	NS				
Nitrite-N	NS	0.744*	NS	0.719*			
Ammonia-N	0.648*	NS	NS	0.804**	NS		
Nitrate-N	NS	0.793**	NS	NS	0.785**	NS	
TOC	NS	NS	NS	0.717*	NS	0.781**	NS
TN	NS	0.842**	NS	NS	0.738**	NS	0.989**

n=10; **p<0.01, *p<0.05, NS = p>0.05; a two-tailed test was used.

RSA= Relative sequence abundance at class level.

Based on the differential analysis, a total of 48 OTUs were identified as showing significant difference of relative abundance (p<0.01) between biofilm and bulk water samples. Among them, 21 OTUs were clustered at the genus level (Table 4).

Table 4 Differential analysis of relative OUT abundance in class level within biofilm and water samples

P-value	Genus
1.16E-04	*Mycobacterium*
1.16E-04	*Clostridium_sense_stricto*
1.25E-04	*Dyadobacter*
1.80E-04	*Flavisolibacter*
2.93E-06	*Aquisphaera*
1.43E-04	*Prosthecobacter*
3.20E-05	*Thiobacillus*
1.19E-04	*Aquabacterium*
1.30E-08	*Azospira*
1.85E-11	*Cupriavidus*
3.01E-07	*Azoarcus*
4.74E-06	*Stenotrophomonas*
5.35E-06	*Pseudoxanthomonas*
5.89E-10	*Hyphomicrobium*
5.19E-07	*Phenylobacterium*
4.78E-07	*Hoeflea*
1.11E-10	*Altererythrobacter*
8.32E-07	*Novosphingobium*
3.55E-08	*Sphingobium*
7.87E-12	*Sphingomonas*
1.22E-03	*Peredibacter*

The alpha-diversity analysis presented both Chao 1 richness estimator and Shannon diversity index of biofilm and water samples. From Fig.4, it can be seen that the richness and diversity within biofilms were all higher than that in the bulk water samples.

Non-metric Multi-Dimensional Scaling analysis (MDS) indicates a clear difference between biofilm and bulk water samples (Fig.5). The results from UniFrac analysis (both Un-weighted and Weighted) also showed a separation based on sample types (Fig.6 and Fig.7).

3.4 The effect of hydraulic regimes on microbial community

In the current study, both biofilm and water samples from different hydraulic regimes shared most of the same components in class level, but the relative abundance differed in most cases. *Planctomycetia* was the predominant group within the biofilms formed under flow rates of 2L/min, 8L/min and 10L/min (36%, 28% and 24% respectively) in test phase 1 (Cl_2/ NH_3-N mass ratio = 3:1) samples. While running at 4L/min and 6L/min, *Alphaproteobacteria* and *Actinobacteria* were abundant (up to 26% and 33% respectively) (Fig.2). However, the structure of the bacterial community differed under most of the hydraulic conditions between test phase 1 and 2. Except in condition with flow rate at 6L/min where *Actinobacteria* remained dominant, the presence of this group increased in cells with flow rate at 4L/min when the chlorine and ammonia nitrogen mass ratio changed to 5:1 in test phase 2 (from 20% to 36%). On the other hand, *Betaproteobacteria* was found to be more dominant in the rest three cells (running at 6L/min, 8L/min and 10L/min) in both test phases (from 6%, 11% and 15% to 27%, 43% and 46%) (Fig.2). *Gammaproteobacteria* and *Cytophagia* were the other main predominant phylogenetic groups within the biofilms from test phase 2.

In the bulk water, the different hydraulic regimes did not clearly influence the composition of the water samples at class level. *Alphaproteobacteria* was predominant in most of the samples (except the cell running at 8L/min in test phase 2), followed by

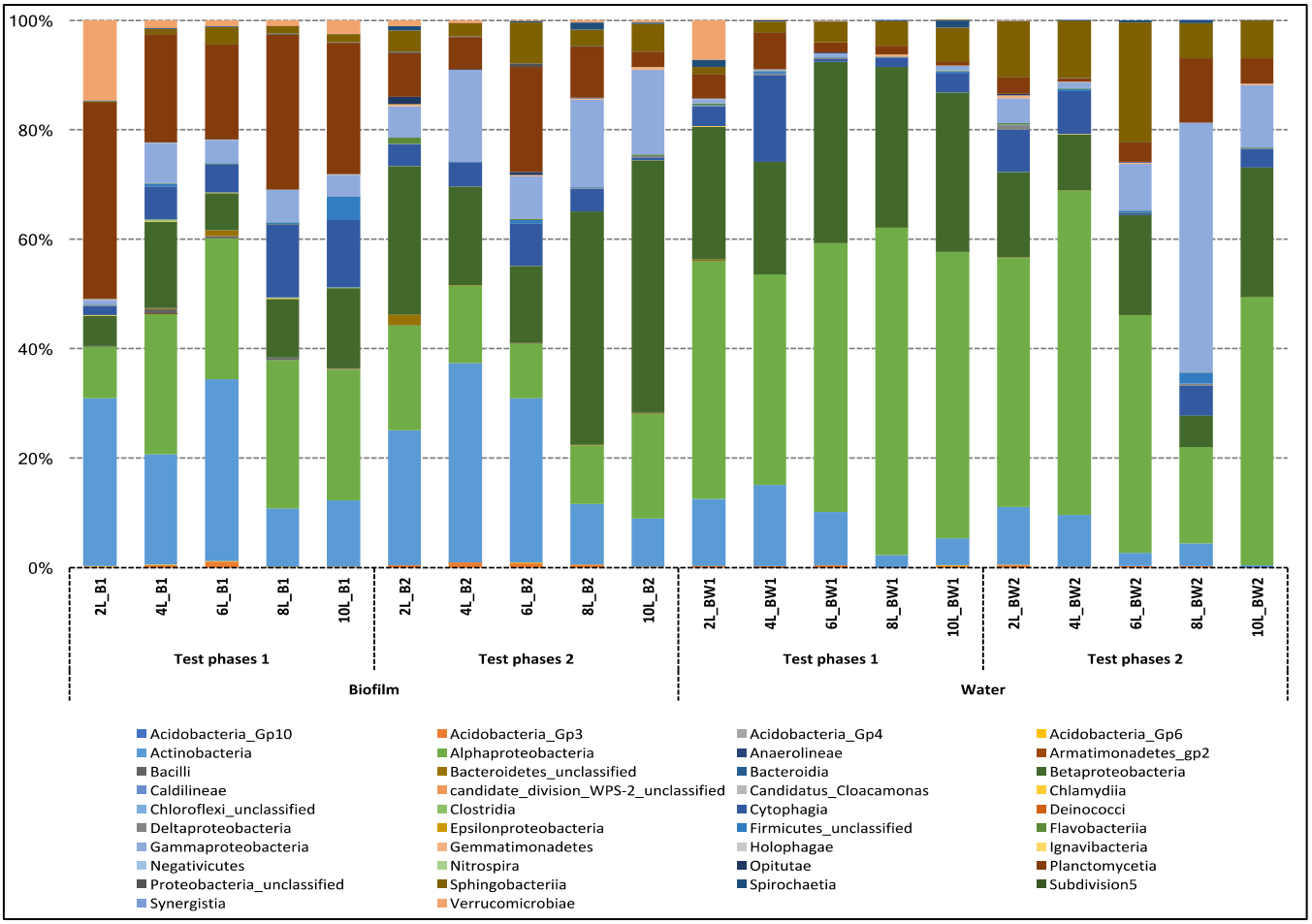

Figure 2 Comparison of the relative abundance of the major phylotypes (class level) found in biofilms and bulk water under the different operation conditions. 2L (flow cell running at flow rate of 2 L/min; B (Biofilm); BW (Bulk water).

Betaproteobacteria, Sphingobacteriia and *Actinobacteria*. Despite the high similarity found in the distribution of bacterial groups in water samples, *Gammaproteobacteria* was observed to be only abundant in the cell running at 8L/min in test phase 2 (up to 46%), where Cl_2/NH_3-N mass ratio was 5:1 (Fig.2).

Mycobacterium was the genus predominant in the composition of most biofilm samples, especially in cells running at 6L/min (total up to 60%). The microbial composition differed in cells running with flow rate at 8L/min between the two test phases, where *Gemmata* and *Azospira* were respectively most dominant (Fig.3). In the biofilms conditioned at 4L/min, 8L/min and 10L/min in test phase 2, *Pseudoxanthomonas* was more abundant when compared with those in biofilms incubated at the other two hydraulic regimes (2 and 6 L/min). The percentages of these bacterial genera were different between hydraulic regimes but did not show a clear trend (Fig.3).

The hydraulic regimes significantly influenced the community composition of bulk water samples at genus level. In most of the conditions, the predominant group differed. In test phase 1, *Sphingobium, Porphyrobacter* and *Sphingomonas* were the most abundant in bulk water from the cells operated at 2L/min, 8L/min and 10L/min (19%, 54% and 40% respectively). *Mycobacterium* accounted for the most predominant group in bulk water from cells at 4L/min and 6L/min (39% and 40%). In test phase 2, *Sphingomonas* was dominant in 2L/min and 4L/min condition (up to 35% and 58% respectively). *Flavisolibacter* accounted for 31% in 6L/min cell and *Legionella* was dominant in 8L/min and 10L/min (up to 56% and 31%) (Fig.3).

The species richness (Chao1 estimator) within the biofilm samples showed a declining trend with increasing flow rate in test phase 2; while flow cell running at 4L/min in test phase 1 showed the highest richness (Fig.4). The diversity (Shannon index) varied under different hydraulic regimes and test phases. In both test phases, the diversity was relatively higher under lower flow rate (2L/min and 4L/min), and it showed an increasing trend with increasing flow rate ranging from 6~10 L/min.

Within bulk water samples, both richness and diversity indicated a higher potential under lower hydraulic regime (i.e. 2~4 L/min), compared with flow rate ranging from 6 to 10 L/min (Fig.4).

The non-metric MDS based on relative abundance of sequence and the un-weighted UniFrac metrics, did not cluster in the distribution of biofilm samples from different hydraulic regimes (Fig.5 and Fig.6). Results from the weighted UniFrac metrics did not show clear patterns in biofilm sample from test phase 1, while for test phase 2, there was a better cluster for biofilm sample

based on hydraulic condition (Fig.7). Compared with biofilm samples, the MDS clearly separated the water samples between the different hydraulic regimes (Fig.5). Despite this, the un-weighted/weighted UniFrac metrics did not clearly cluster water samples based on the hydraulic regimes (Fig.6 and 7).

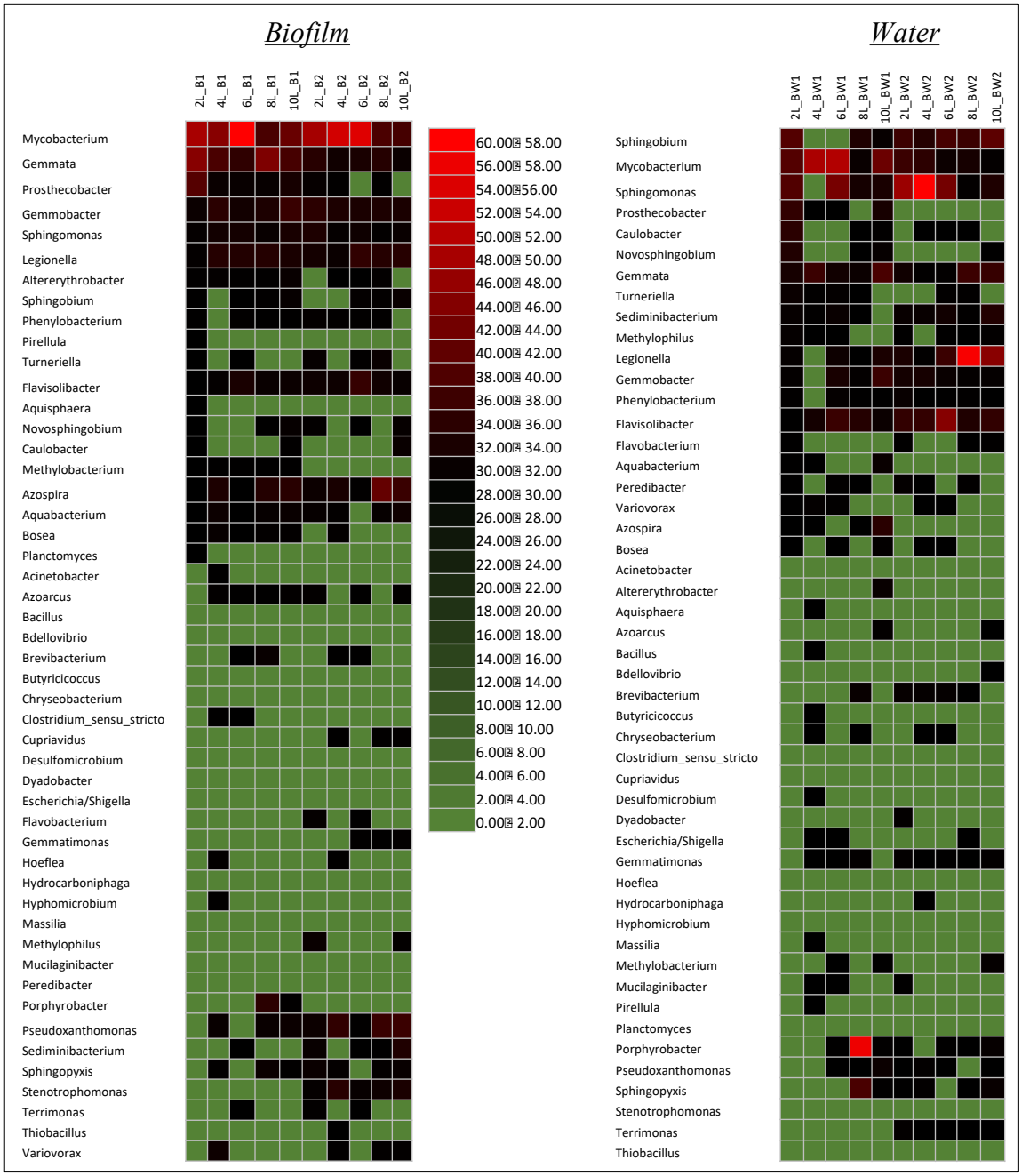

Figure 3 Heatmaps show the percentages of most abundant species at genus level within bulk water and biofilms from flow cells running at different conditions. 2L (flow cell running at flow rate of 2 L/min; B (Biofilm); BW (Bulk water).

3.5 The effect of Cl_2/NH_3-N mass ratio on microbial community

There were differences in the bacterial community composition between both biofilm and bulk water samples from the different Cl_2/NH_3-N mass ratio states (test phases 1 and 2). This is reflected in the different percentages of relative sequence abundance detected at different phylogenetic levels (Fig.2 and Fig.3). In the biofilm samples, *Planctomycetia* percentage tended to be smaller in the 5:1 state (test phase 2). The presence of *Betaproteobacteria* and *Gammaproteobacteria* was greater in the 5:1 state in all hydraulic conditions. The difference of *Betaproteobacteria* abundance was remarkable, and it was 6%, 16%, 7%, 11% and 15% at each flow condition (2L/min, 4L/min, 6L/min, 8L/min and 10L/min respectively) in the 3:1 state; and 17%, 18%, 14%, 43% and 47% in the 5:1 state (Fig.2). At the genus level, the abundance of certain bacterial within biofilm also differed (Fig.3). For example, the percentage of *Gemmata* that was accounted for was much greater in biofilm samples in the 3:1 condition than that

in the 5:1 state (i.e. 31% at 2L/min in 3:1 state, while it was 9% in 5:1). At 4L/min, 8L/min and 10L/min conditions, *Pseudoxanthomonas* was relatively higher in the 5:1 state (11%, 13% and 14% respectively), where there were all around 2% in the 3:1 state. The structures of microbial composition at 6L/min in each disinfection condition were similar at genus level.

Within bulk water samples, different mass ratios did not significantly affect the community composition at class level (Fig.2). Only when the flow cell was run at 8L/min, *Gammaproteobacteria* was the most dominant in the 5:1 state (up to 46%), while it was only 0.07% in the 3:1 state. The abundance of *Alphaproteobacteria* and *Betaproteobacteria* was quite smaller in 5:1 (18% and 6% respectively) when compared with that in the 3:1 state (60% and 29% respectively) (Fig.2). At genus level, the community composition at all hydraulic conditions in the two states were remarkably different (Fig.3). For instance, *Mycobacterium* was the most dominant at 4L/min in the 3:1 state (up to 39%), while it was only 10% in the 5:1 state. *Sphingomonas* was negligibly small in

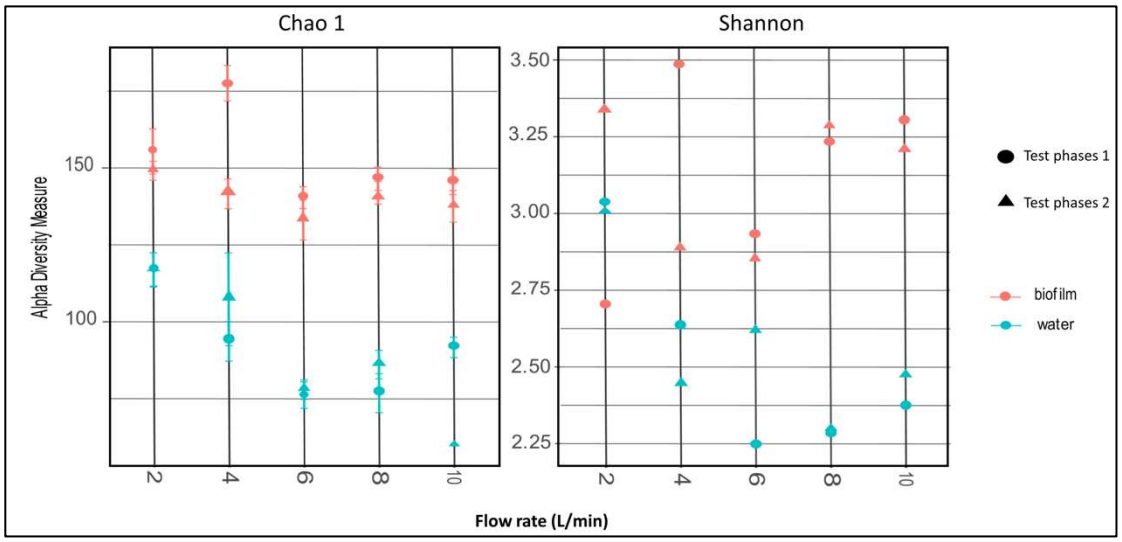

Figure 4 Alpha-diversity results for both biofilm and bulk water samples under different operation conditions

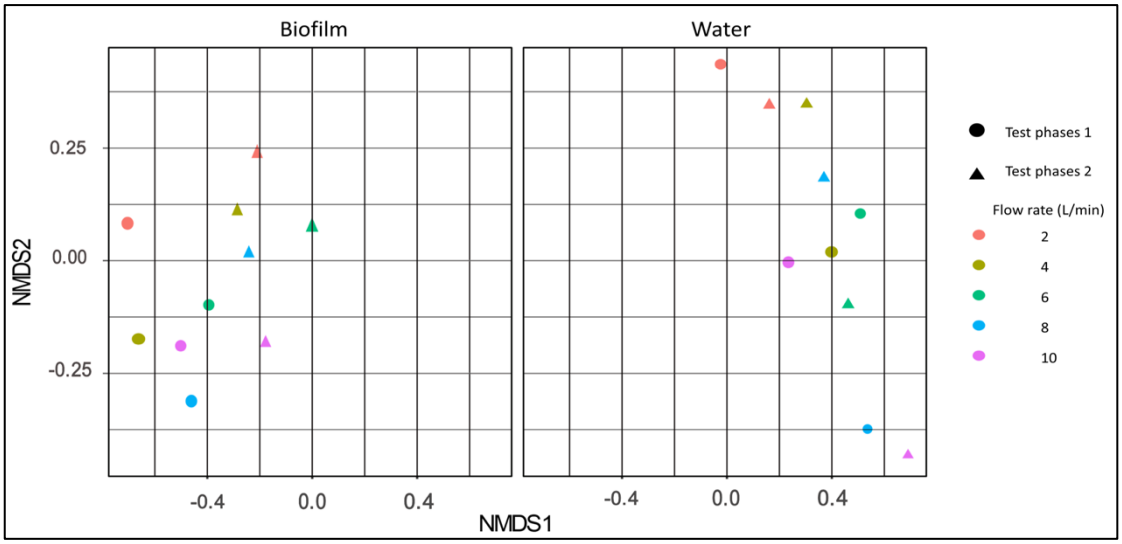

Figure 5 Two –dimensional plot of the Multi-Dimensional Scaling (MDS) analysis based on Bray-Curtis similarities of the percentage sequence abundance. Symbols and colour representing individual samples and sample type.

the 3:1 state (around 0) and was the predominant species in the 5:1 state (up to 58%). The difference in the bulk water samples in cells at 8L/min and 10L/min was also obvious. *Porphyrobacter* and *Mycobacterium* were predominant within samples in the 3:1 state (54% and 24% respectively), while their percentage were only 0.1% and 1% in the 5:1 state. Under these two flow conditions, *Legionella* accounted for the largest percentage (56% and 31% respectively) in both flow conditions when the Cl_2/NH_3 mass ratio was 5:1, and this was quite small in 3:1 state (0.07% and 6%).

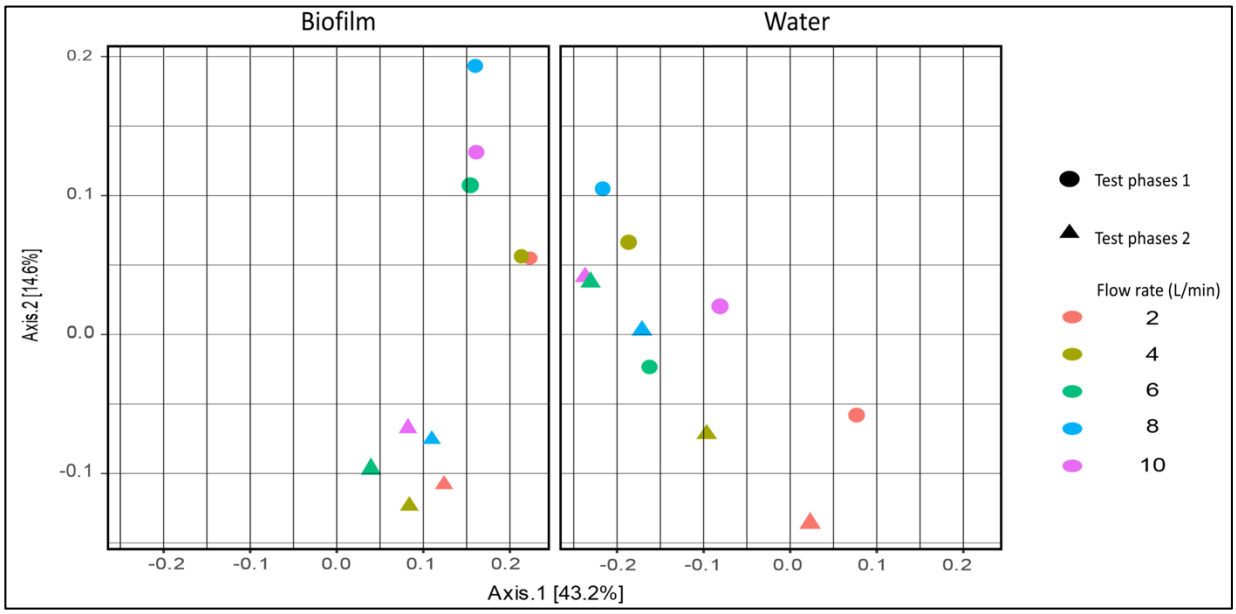

Figure 6 Two dimensional coordinates plots of Un-Weighted UniFrac analysis (n = 20) showing the phylogenetic clustering of bacterial communities within both biofilm and water samples at 97% similarity. The axes are scaled based on the percentage of variance that they are explaining.

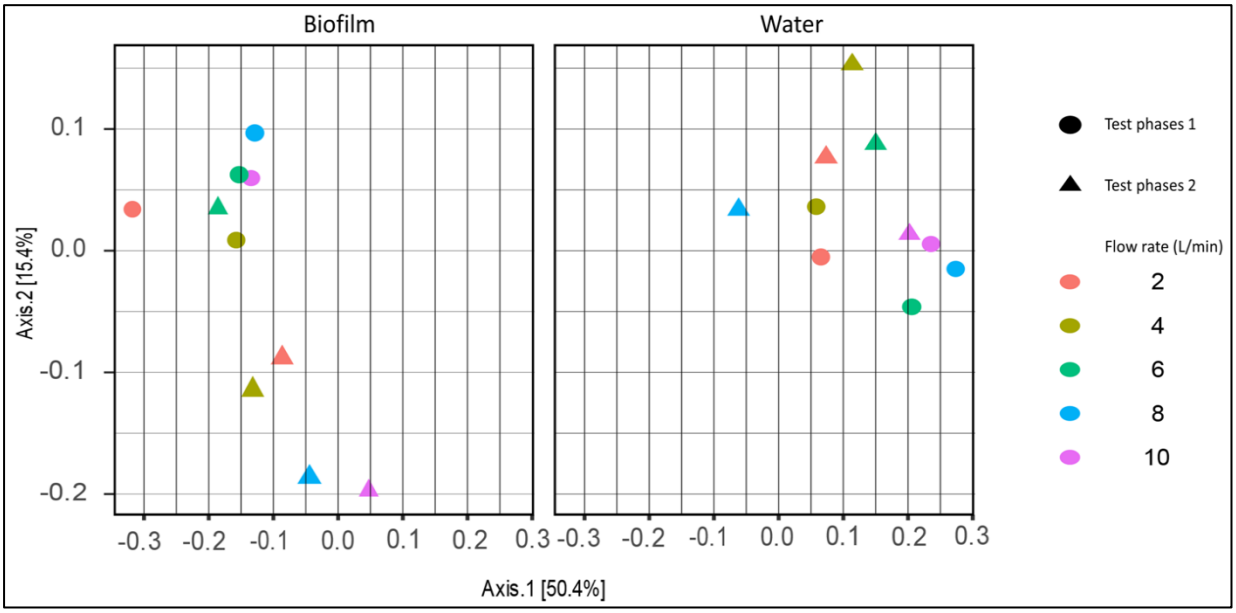

Figure 7 Two dimensional coordinates plots of weighted UniFrac analysis (n = 20) showing the phylogenetic clustering of bacterial communities within both biofilm and water samples at 97% similarity. The axes are scaled based on the percentage of variance that they are explaining.

There was no significant difference in species richness (Chao 1 index) between the two test phases under most of the hydraulic conditions (Fig.4). Only slight differences were identified in biofilm samples from flow cell running at 4L/min and also bulk water samples at 10L/min condition. For species diversity (Shannon index), only biofilm samples from cells with flow rate of 2 and 4 L/min presented differences according to the Cl_2/NH_3-N mass ratios. Nether richness nor diversity variation followed a clear trend.

The MDS analysis clearly clustered the biofilm sample from the two test phases based on Cl_2/NH_3-N mass ratio (Fig.5). There was a significant difference in the community composition within biofilm samples according to Cl_2/NH_3-N mass ratio within un-weighted and weighted UniFrac metrics (Fig.6 and Fig.7). However, no clear separations in the distribution of water samples based on test phases were observed (Fig.5, 6 and 7).

4. Discussion

Difference in microbial community between biofilm and bulk water sample was identified according to dissimilarity analysis and UniFrac metrics (Fig.5 ~Fig.7). The alpha- diversity analysis also indicated higher species richness and diversity within biofilm when compared with that in bulk water samples (Fig.4), and this is in agreement with the results from a pilot-scale experimental DWDSs[1]. Previous work has shown that some bacterial community presented better ability for attaching to material and forming biofilm[42,43]. These bacteria could produce more high-quality polymers to form biofilm and hence increase the capacity to withstand the hydraulic attack. As a result, the biofilm which can work as a shelter for protecting bacteria from outer interference such as low nutrient, disinfectant and flushing, attracts more bacteria to accumulate[4]. This might explain the difference in bacterial community and diversity between biofilm and bulk water.

In order to control water quality and to prevent possible microbial contamination through DWDSs, water utilities are expected to apply disinfectant to the water before it enters the system, and to also maintain the disinfectant residual at a reasonable level. Chlorine and chloramine are the two main disinfectants used worldwide. In particular, the application of chloramine has increased due to its low contribution to the formation of disinfectant by products (DBPs)[44]. In the current study, chloramine was applied as the disinfectant. According to previous studies, bacterial groups showed different sensitivity to disinfectant[45,46]. The results from the current study are in agreement with researches on microbial structure detected in systems with chloraminated water[2,47-49], where *Alpha-* and *Betaproteobacteria* were dominant in biofilm and water samples (Fig.2). In addition, *Actinobacteria* and *Planctomycetia* were also the abundant bacterial groups identified in biofilm samples in the current study. Krishna *et al.* (2013) observed that the former group dominated in high chloramine containing water system and this group is a major class that contains possible denitrifiers[51]. The high abundance of this group detected within the current study might provide a clue that why there was a decline of nitrate nitrogen concentration in all flow cell units (Table 2). The latter group (*Planctomycetia)* was also found in annual reactor fed with low concentration of NH_2Cl (0.06mg/L)[2].

At genus level, unlike chlorinated systems where *Pseudomonas* was always abundant[1,52], *Mycobacterium* was dominant (17%~60%) within biofilm samples in the current study (Fig.3). This is in agreement with the results in previous studies about bacterial community in monochloramine-treated drinking water biofilms[46,53]. In addition, high presence of species relative to *Mycobacterium* were also detected in biofilm from chloraminated DWDSs[54,55]. Other genera that dominated within the current study was *Sphingomonas*, which has been reported to be abundant in chloraminated environment[49,50,56]. Moreover, compared with chlorinated systems, this group present the ubiquity in chloramine-treated systems[48], and it was considered as an indicator of the onset of nitrification[50]. *Legionella* was also observed to be predominant within the current study. This group is always considered as the opportunistic pathogens, and it is the most frequent causative agent of drinking water related disease outbreaks[57]. Other abundant genera species in this study, such as *Gemmata* and *Porphyrobacter* have been observed in drinking water samples and suggested to be adapted to oligotrophic conditions in DWDSs[58-60]. In addition, *Azospira*, which belongs to denitrifying genus[61], was detected to be abundance as well. Combine with the discussion above, the decrease of nitrate nitrogen in this study could due to the possible occurrence of denitrification process.

The bacterial community composition and structure of biofilm and water samples differed between the different hydraulic regimes (Fig.2 and Fig.3). However, only statistical difference was observed within water samples (Fig.5). The consistency of the biofilm samples might be expected since the biofilm within each flow cell unit was firstly developed at the same condition before the test, and a common recirculation tank was used for all cells. The bacterial structure in biofilm was more stable and resistant to the change of outer environment than that in water, although distinctive microbial community within biofilm was observed under similar developing conditions[62]. In addition, as the biofilm samples in current study were only collected at the end of the test, and the sequencing technology used only detected possible organisms without differentiating the live/dead status, further research in terms of monitoring microbial community over time is needed. In contrast with the results observed by Douterelo *et al.* (2013) who found similar community composition in water under different hydraulic regimes, the microbial community in water sample was clustered separately between different flow rate conditions in the current study (Fig.5). This phenomenon might be explained by the observation in previous studies that hydraulic condition showed effects on the material build up and subsequent mobilization within pipe-scale DWDSs[63-65]. Sekar *et al.* (2012) also provided evidence by analysing bacterial abundance and structure from real WDS and suggested that the bacterial composition varied and was possibly associated with system hydraulics.

The alpha- diversity analysis provided comparison of species richness and diversity between different hydraulic regimes and the results reveal that both richness and diversity tended to be higher at lower flow rate conditions (Fig.4). In the test phase 1, both the highest species richness and diversity occurs in biofilms conditioned at 4 L/min. On the other hand, the species diversity within biofilm presented an increasing trend at flow rate ranging from 6 ~ 10 L/min. The high richness and diversity in biofilms from lower flow rate condition was potentially due to a less survival pressure from the damage caused by excessive shear stress. In addition, previous studies have suggested that higher flow might favour the development and growth of biofilm due to the promotion of transport and diffusion of nutrient within biofilms at high velocities[67]. This may explain why there was an increasing trend in diversity associated with the increase of flow rates within current study. In contrast, both Rochex *et al.* (2008) and Rickard *et al.* (2004) reported a decrease of biofilm diversity under higher shear stress, and this might slow down the process of biofilm maturation. The promotion and inhibiting effects from increasing shear stress on biofilm structure might work interactively, and hence result in the variation between studies. However, the cited research was undertaken in annual reactors in which nutrient and operational conditions varied with each other. Consequently, there was not enough evidence to support how the microbial community in the biofilm respond to different hydraulic conditions in real systems.

The species richness and diversity were observed to be higher within water samples at lower hydraulic regimes (Fig.4). This trend was expected to be as a result of interaction between biofilm mechanical properties and hydraulics. Studies have suggested that a more dense and compact biofilm would develop under higher shear stress condition, and greater detachment force was then required to remove bacterial material[70,71]. Similar observation was noted by Vrouwenvelder *et al.* (2010) who found that biofilm formed under lower shear stress condition was easily removed. Sharpe *et al.* (2010) also reported less material mobilized to bulk water under higher conditioning shear stress and there were materials remaining on pipe coupons even after flushing events. Consequently, biofilm developed under higher flow rate condition in the current study resulted in less microbial material mobilized into the bulk water.

The current study used chloramine as disinfectant and investigated two Cl_2/NH_3-N mass ratio (3:1 and 5:1) in two separate test phases. The results from similarity analysis and UniFrac matrix suggest a difference in composition and structure of biofilm samples between the two ratios (Fig.5 ~ Fig.7). The difference between these two mass ratios was that there was excessive ammonia when monochloramine was prepared with smaller ratio (3:1). Lee *et al.* (2011) used microelectrodes to monitor the penetration of disinfectant and dissolved oxygen into nitrifying biofilm developed in an annual reactor. The author suggested that the excessive ammonia would further promote the chemical decay of chloramine and hence accumulate more free ammonia. This excessive ammonia would affect the penetration of chloramine and DO into the biofilm, where the disinfectant was impeded and the oxygen was consumed by free ammonia[27]. Based on the information, the microbial activity and the level of inactivation by disinfectant within biofilm would be influenced when compared with the system using larger mass ratio (5:1). However, the author did not analyse the microbial composition after disinfection. In addition, even though the biofilm was developed under the same condition before the two test phases, the bacterial composition and structure might be different from each other. Furthermore, without working as a single influencing factor, interaction between hydraulic regimes and disinfection strategy might cause the difference in microbial community within biofilms from these two test phases. Further research into microbial succession in biofilms within the current experimental facility is required to verify the impact of Cl_2/NH_3-N mass ratio on microbial composition and structure.

Although the onset of nitrification was observed based on physico-chemical analysis within the current study (Table 2 and Fig.S5), few nitrifier (AOB/NOB) related sequences were detected (Fig.2 and Fig.3). Only small relative abundance of *Nitrospira* (< 0.01%) was classified in three biofilm samples in test phase 1. This low rate of detection might be due to the fact that the nucleus of this community available for sequencing was limited, and also the sequencing depth was not enough to acquire sufficient information. Sawade *et al.* (2016) used both MiSeq and qPCR to detect microbial community within onset of nitrification batch test, and the results suggested a very low fraction of nitrifier detected from MiSeq sequencing even in system with high production of nitrite. In comparison, the qPCR was relatively sensitive and the results indicated correlations between community abundance and nitrification[74]. In order to characterize the AOB and NOB within chloraminated DWDS, Regan *et al.* (2002) applied terminal restriction fragment length polymorphism (T-RFLP) analysis and this technology indicated the occurrence of related sequence successfully. Therefore, in order to better understand the relationship between operational conditions and abundance of nitrification related microbial community, techniques targeting particular species such as qPCR and T-RFLP are needed in further research. However, despite nitrifiers, *Planctomycetia,* which belongs to the group of anammox bacteria was identified dominant within biofilm samples (Fig.2). This kind of bacteria is also considered as an important participant in nitrification process, as they could convert ammonium and nitrite directly into nitrogen gas under anaerobic condition[75]. Although the current experimental condition was measured as aerobic by testing dissolved oxygen within water tank (Table S2), anaerobic environment might occur at the inner part of the biofilm due to low oxygen penetration rate into biofilm[76].

The bacterial composition results have confirmed that even under limited nutrient conditions, the drinking water system could still be a robust ecological niche for microbes. Moreover, opportunistic pathogens including *Legionella* and *Mycobacterium* were observed to be abundant within the current study. From the physico-chemical parameter analysis, there was a dramatic decline of disinfectant in all the flow cell units (Table 1). On one hand, this low disinfectant residual was due to a three-day water age which would increase the level of disinfectant auto-decomposition and allow for a long reaction time between disinfectant and existing water chemicals[77,78]. The onset of nitrification process was another key factor that accelerated the decay of chloramine. Both Krishna *et al.* (2013) and Sathasivan *et al.* (2008) monitored a high chloramine decay rate along with the nitrification process. The excessively high disinfectant decay rates would reduce the impact from outer environment on the growth of bacteria, and consequently increase the chance of appearance of opportunistic pathogens.

Both the increase of nitrite concentration during the tests and the detection of *Sphingomonas* in the current study indicate the onset of nitrification within systems[50]. The pathogens observed in current study further confirmed the importance of maintaining disinfectant residual and controlling nitrification within DWDSs. Based on the results from this study, neither high shear stress nor large Cl_2/NH_3-N mass ratio was an effective approach to maintain water quality in system with the onset of nitrification. However, it is still important to understand the microbial community and structure within systems under different operational conditions. Such information could assist in investigating the relationship between bacterial growth and environmental factors, and improve the effectiveness of management strategy by providing microbial indicator of water quality. Further research, using target sequencing technology and monitoring the community composition over time will help to better understand the occurrence of bacteria in different operating conditions, and to develop maintenance strategy for securing public health.

5. Conclusions

Nitrification in chloraminated DWDSs has received much consideration due to its impact on water quality and public health. However, there is less research conducted on investigating microbial community under different hydraulic regimes and Cl_2/NH_3-N mass ratios in systems experiencing the onset of nitrification. This paper presents results of application of high throughout Illumina MiSeq analysis to chloraminated experimental flow cell systems, which yields new and unique data about the impact of hydraulic regimes and Cl_2/NH_3-N mass ratio on bacterial community composition and structure in biofilms and bulk water. The outcomes of this study are summarized below:

- The bacterial community composition and structure were different between biofilm and bulk water. This difference suggests that microorganism within biofilm presented better capacity to produce high resistance polymers to form biofilm. On the other hand, the bacterial groups identified within the bulk water were different to those found in chlorine water DWDSs, and it was expected that these groups have better resistance to chloramine.
- Overall, species richness and diversity in biofilm tend to be higher at lower flow rates, while the diversity increases with the increase of shear stress when the flow rate is between 6 and 10 L/min. This suggests the uncertainty of hydraulic effects on biofilm development.
- There was no statistical difference in microbial structure identified in biofilm between different hydraulic regimes and this suggested the stability of biofilm to outer environment.
- Different hydraulic regimes affect the bacterial community composition and structure within bulk water, with a tendency of higher richness and diversity detected at lower hydraulic regimes. This confirms the influence of hydraulic condition on biofilm mechanical structure and further material mobilization to water.
- Cl_2/NH_3-N mass ratio showed obvious effect on microbial structure in biofilm, suggesting excessive ammonia would be a factor affecting chloramine penetration to biofilm and the microbial activity within biofilm.
- Opportunistic pathogens such as *Legionella* and *Mycobacterium* were detected in abundance in the experimental system. This confirms that biofilm could be a suitable reservoir for these microorganisms and further suggests that nitrification can lead to decrease of water quality and microbial outbreaks.

Conflicts of interest

There are no conflicts to declare.

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